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Study of Merkel cells in the dog through the immunohistochemical expression of five different commercial antibodies: comparative analysis

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ABSTRACT

Merkel cells (MCs) are cutaneous specialized cells with a wide immunohistochemical profile, including low molecular weight cytokeratins and various endocrine and neural markers. Differences in expression of these markers may be a consequence of contradictory results in studies of MCs in normal and damaged canine tissues. The present study aimed to compare five different commercial available antibodies developed against cytokeratins 8 + 18 and 20, neuron-specific enolase, chromogranin A, and synaptophysin on formalin-fixed paraffin-embedded samples of hard palate, cheek skin including sinus hair follicles or vibrissae, nasal planum, and footpads. The antibodies showed great variability with respect to quality and intensity of immunoreactivity to identify MCs. Anti-Cytokeratin 20 antibody is more effective to recognize MCs in the dog skin followed by the antibodies against neuron-specific enolase, cytokeratins 8 and18, chromogranin A, and synaptophysin. There was also a significant difference in intensity of immunoreaction scores between them depending on the location examined. These results represent a necessary basic background for future studies of the role of these cells in normal and damaged canine tissues.

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Introduction

Merkel cells (MCs) were first described in 1875 by Friedrich Sigmund Merkel as 'tastzellen' (touch cells). These specialized clear cells function as slowly adapting type-I mechanoreceptors and are concentrated in touch-sensitive areas in glabrous and hairy skin and in ectoderm-derived mucosa (Merkel 1875; Halata et al. 2010; Maksimovic et al. 2014). In addition, MCs are believed to be involved in paracrine functions related to skin homeostasis and cutaneous nerve development (Tachibana 1995; Boulais et al. 2009). They contain a plethora of active molecules known to be neurotransmitters, biogenic monoamines, endocrine and neurocrine substances, neurotrophins, or growth and differentiation factors for various cell types, such as neuron-specific enolase (NSE), synaptophysin (SYN), chromogranin A (CGA), vasoactive intestinal polypeptide, bombesin, met-enkephalin, serotonin, pancreastatin, substance P, peptide histidina isoleucine, and calcitonin gene-related peptide (Gauweiler et al. 1988; Hartschuh et al. 1989; Fantini & Johansson 1995; Tachibana 1995). These findings justified the classification of mammalian MCs with the diffuse neuroendocrine system. On the other hand, MCs also express cytokeratin polypeptides demonstrating their epithelial cell character, including low molecular weight cytokeratins (CK) 7, 8, 18, 19, and 20 (Moll et al. 1984, 1995; Narisawa et al. 1992; Lundquist et al. 1999; Eispert et al. 2009).

Since the immunohistochemical profile of canine MCs also appears to be wide (Ramírez et al. 2014), it would be of interest to study whether differences in the identification and number of canine MCs could be found using different available commercial antibodies in the same tissues. The selection of the most sensitive and specific antibody is crucial to the selection of the most appropriate markers for diagnostic purposes and to perform studies of density and anatomical mapping in cell populations. The aim of the present study was to compare the immunohistochemical expression of five commercially available monoclonal and polyclonal antibodies developed against low molecular weight cytokeratins and neuroendocrine molecules, in order to determine possible differences in terms of immunoreaction and counting of MCs that allow selecting the most reliable antibody for study of density and distribution of MCs and their neoplasms in the dog.

Material and methods

Sample selection and tissue preparation

Five, male and female, healthy young adult dogs were used for the study. They had been humanely euthanized and destined for academic training according to current legislation (RD53/2013 from Spanish Government and 2010/63/UE Policy from European Union on the Protection of Animals Used for Scientific Purposes). Samples from body locations reported to be MC-rich areas (Tachibana 1995; Halata et al. 2010) were collected: hard palate, cheek skin including vibrissae, nasal planum, and

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footpads. The macroscopic appearance of the tissues included had been normal. Specimens were fixed in 10% neutral buffered formalin, dehydrated through graded alcohols, and embedded in paraffin wax. Control samples were also obtained from autopsies, fixed in 10% neutral buffered formalin, and embedded in paraffin wax (Table 1). Sections that were 3 µm thick from each location were stained with haematoxylin and eosin (HE) and were examined to corroborate the absence of pathologic changes.

Antibody specificity testing and optimization

Samples from oral mucosa and positive control tissues were used for assay optimization. Five different commercial antibodies were chosen for comparative analysis (Ks20.8, NCL-5D3, BBS/NC/VI-H14, anti-CgA, and SY38 antibodies; for a detailed description see Table 1) and were tested with a rigorous set of varying assay conditions, including different antigen retrieval methods (water bath-heating in citrate-based buffer at 80, 90, and 95°C, microwave-heating in citrate-based buffer at 15, 20, and 30 min, either alone or in combination with digestion by proteinase-K at 5, 7, and 10 min), antibody dilutions (ranged from 1:10 to 1:5000), and antibody time of incubation (ranged from 1 h to overnight/18 h) in order to determine the optimal conditions to enhance each antibody's performance. Upon determination of preliminary assays, staining patterns in control and test tissues were compared.

Progressive iterative steps were employed based on the results of prior staining runs to identify the conditions that demonstrated accurate cellular localization of each antigen, an appropriate signal-to-noise ratio, and acceptable performance in positive and negative tissue controls.

Immunohistochemical protocol

Serial tissue sections that were 3 µm thick were collected onto Vectabond-coated slides (Sigma Diagnostics, St Louis, MO, USA), incubated at 37°C for 24 h, deparaffinized, rehydrated in graded alcohols, and incubated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. The sections were then subjected to heat-induced antigen retrieval by placing in 1 mmol/litre ethylenediaminenetetraacetic acid (Sigma-Aldrich Chemical Co., St Louis, MO, USA) at pH 6.0 in a water bath at 95°C for 30 min. After cooling, the slides were covered with 10% normal rabbit (for monoclonal antibodies) or porcine (for polyclonal antibodies) sera in phosphate buffered saline (PBS) for 30 min at room temperature before incubation with the primary antibodies for 18 h at 4°C. Technical data of primary antibodies are listed in Table 1. Expression of these antibodies by MCs had been previously tested (Ramírez et al. 2014).

A biotinylated rabbit anti-mouse immunoglobulin G (IgG; Dako, Glostrup, Denmark) diluted 1:200 in PBS containing 1% normal rabbit serum (for monoclonal antibodies) or a biotinylated porcine anti-mouse immunoglobulin G (IgG; Dako) diluted 1:200 in PBS containing 1% normal porcine serum (for polyclonal antibodies) was applied for 30 min at room temperature as secondary antibody. The avidin-biotin-peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA, USA) was then applied as labelling and amplification reagent. To bring out the immunoreaction, 3-amino-9-ethylcarbazole (AEC) (Sigma-Aldrich Chemical Co.) was applied to the slides for 3 min at room temperature. Sections were lightly counterstained with Mayer's haematoxylin, washed in tap water, and aqueous mounted (Shandon Immu-Mount™, Thermo Electron Corporation, PA, USA).

Evaluation of immunohistochemistry

Positive immunoreactivity was characterized by the presence of a distinct cytoplasmic red reaction without background staining. The immunostaining was evaluated separately and results based on the consensus of board-certified pathologists (GAR, AE). The numerical frequency of MCs was assessed by counting the number of positive cells per 1 cm length of epidermis at 400× magnification. Cells were registered when the nucleus-containing cell body was visible on the microscopic field, while cytoplasmic processes or partial cross-sections were discharged. Results were expressed as number of *MCs per centimetre of basal zone* (MCs/cm). The intensity of immunoreaction for every positive cell was evaluated at 1000x magnification and reported in a four-point scale (0 = no expression, 1 = mild expression, 2 = moderate expression, 3 = strong expression). Mean values were obtained for each location under study. Positive control tissues (Table 1), along with hematoxylin and eosin-stained and negative reagent control slides, were used as an aid to the scoring in every assay.

Statistical methods

Statistical analysis was performed using the IBM SPSS® 20 software package (IBM Corporation, Armonk, NY, USA). For the quantitative parameters, the mean and standard deviation (SD) were analysed. The number of positive cells and the intensity scores were compared between the five antibodies by one-way ANOVA test. Statistical significance was accepted at $p < .05$. Tukey's HSD (honest significant difference) test was used in conjunction with the ANOVA (*post hoc* analysis) to find means that are significantly different from each other.

Results

MCs were not differentiated from other epidermal clear cell populations by HE stain. Some cells were believed to be MCs by their morphology and location in sinus hair follicles. Round or oval MCs were detected in 98/100 tissue sections by immunohistochemistry. They were placed individualized or clustered in various arrangements at the tips of epithelial pegs of the palatine mucosa, rete ridges of glabrous skin of the nasal planum and footpads, and at the base of the epidermal discs and within the outer root sheath of the hair follicles in cheek hairy skin. Several patterns of cytoplasmic immunoreactivity were noted depending on the antibody used; nuclei were not stained with any antibody used. CK20 and CK8 + 18 presented a similar pattern of immunostaining, predominantly

Table 1. Technical data of antibodies and immunohistochemical procedures.

Antibody[a]	Clon	Type	Manufacturer[b]	Ag retrieval	Dilution	Positive controls
CK20	Ks20.8	M	Dako	HIER	1:40	Human and canine intestinal epithelium
CK8 + 18	NCL-5D3	M	EuroDiagnostica	HIER	1:20	Canine sweat gland epithelium
NSE	BBS/NC/VI-H14	M	Dako	HIER	1:1000	Canine brain
CGA	–	P	Dako	HIER	1:50	Human and canine colon
SYN	SY38	M	Dako	HIER	1:20	Canine adrenal medulla

Note: M = monoclonal; P = polyclonal; Ag = antigen; HIER: Heat-induced epitope retrieval.
[a]See text for abbreviations.
[b]Dako Labs., Glostrup, Dinamarca; EuroDiagnostica Labs., Malmö, Sweden.

diffuse, and homogeneously distributed throughout the cell cytoplasm (Figure 1(a, b)). Occasionally, granular or peripheral cytoplasmic staining was noted. The staining pattern with the anti-NSE antibody was light granular and homogeneously distributed in the cytoplasm (Figure 1(c)). Dermal immunostained nerve fibres penetrated the basement membrane and were closely associated with many immunostained cells. CGA was expressed in a predominantly granular pattern with the strongest immunoreactivity on the basal side of the epidermal and mucosal MCs or on the side facing the follicle in MCs of vibrissae (Figure 1(d)). The immunoreactivity for SYN presented as finely granular immunostaining localized in the superficial face of the cytoplasm in the epidermal and mucosal MCs whereas it was situated in the dermal face in the MCs of vibrissae (Figure 1(e)). Some nerve bundles and perivascular immunostained nerve fibres in dermis were also positive for CGA and SYN antibodies.

The intensity scores and counting of immunopositive cells are summarized in Tables 2 and 3, respectively. Differences in the intensity of immunoreaction for each antibody were noted between MCs in the same or in different locations in the same individual. Acceptance of the assay was based on the consistency in staining patterns and a coefficient of variation (CV) among sample scores that does not exceed 20%. In hard palate and nasal planum, the strongest immunoreaction was obtained for CK20 (2.5 ± 0.4 and 2.5 ± 0.3 scoring, respectively), followed by NSE (2.3 ± 0.6 and 2.3 ± 0.4), CK8 + 18 (1.7 ± 0.4 and 1.5 ± 0.4), CGA (1.5 ± 0.6 and 1.5 ± 0.6), and SYN (1.4 ± 0.4 and 1.3 ± 0.4). In cheek skin and footpads, the intensity of immunoreaction was very similar for CK20 (2 ± 0.4 and 2.3 ± 0.3 scoring, respectively), NSE (2 ± 0.4 and 2.3 ± 0.4), and CGA (2.3 ± 0.5 and 2.3 ± 0.4), and stronger than CK8 + 18 (1.9 ± 0.5 and 1.8 ± 0.3) and SYN immunostaining (1.4 ± 0.3 and 1 ± 0.3) (Figure 1). These scores were found to be statistically comparable. In the hard palate, differences in intensity of immunoreaction were statistically significant between CK20 and CGA ($p = .03$), and CK20 and SYN ($p = .01$). In the cheek skin, only significant differences were noted between CGA and SYN ($p = .02$). Differences in immunoreaction scores were also present in the nasal planum for CK20 compared with CK8 + 18 ($p = .04$), CGA ($p = .04$), and SYN ($p < .01$), and for NSE compared with SYN ($p = .02$). Finally, difference in reaction intensity with SYN was highly significant when compared with CK20 and CGA scores ($p < .01$) in the footpads. The CV for each sample set and antibody is presented in Table 2. Only the antibody anti-CK20 showed consistently a CV $\leq$ 20% (average 12–20%).

Our results also reveal variations in density of MCs between locations and antibodies used. For every location, the highest numbers of MCs/cm of basal zone were consistently obtained with anti-CK20 antibody (Table 3). These differences in MC numbers between the antibodies tested were not statistically significant, excepting in footpads between CK20 and SYN ($p < .01$), NSE and SYN ($p = .03$), and CGA and SYN ($p = .02$).

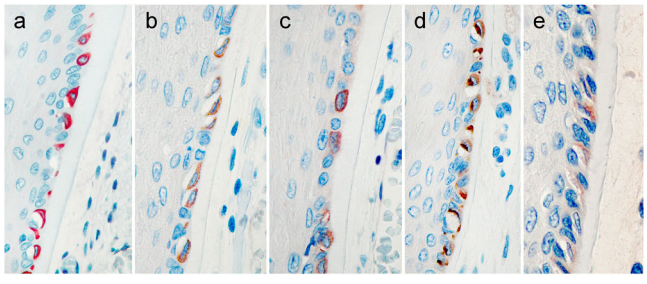

Figure 1. Serial histologic sections of vibrissa showing cytoplasmic immunoreactivity in the MCs for CK20 (a), CK8 + 18 (b), NSE (c), CGA (d), and SYN (e) antibodies. ABC immunohistochemical staining. 400×.

Discussion

The highest numbers of MCs were identified using the anti-CK20 antibody for each location under study. Differences up to 2-fold in the MCs-counting were found for the same anatomical location between the markers, especially between the anti-CK20 and anti-SYN antibodies. In addition, intensity of

Table 2. Mean values (±SD) of intensity of immunostaining for each location with the antibodies used.

		Animals						
ATB	Locations	1	2	3	4	5	Mean ± SD	CV (%)
CK20	Hard palate	2.5	1.8	3	2.8	2.6	2.5 ± 0.4	16
	Nasal planum	2.9	2.3	2.9	2.6	1.6	2.5 ± 0.3	12
	Cheek skin	2	1.7	1.8	2.8	1.6	2 ± 0.4	20
	Footpads	2.1	2.2	2	2.8	2.5	2.3 ± 0.3	13.1
CK8 + 18	Hard palate	1.7	2	1	1.7	2	1.7 ± 0.4	23.5
	Nasal planum	1.3	1.6	2	1.7	1	1.5 ± 0.4	26.7
	Cheek skin	2.3	1.5	2.3	2.2	1.2	1.9 ± 0.5	26.3
	Footpads	1.6	2	2	1.3	2	1.8 ± 0.3	16.7
NSE	Hard palate	2.7	1.3	2.6	2.7	1.9	2.3 ± 0.6	26.1
	Nasal planum	2	2.1	2	2.6	2.8	2.3 ± 0.4	17.4
	Cheek skin	1.7	1.5	2.5	1.8	2.5	2 ± 0.4	20
	Footpads	2.3	1.9	0	2.5	2.5	2.3 ± 0.4	17.4
CGA	Hard palate	2	1.2	1	1.2	2.3	1.5 ± 0.6	40
	Nasal planum	2.4	1	1	1.9	1.3	1.5 ± 0.6	40
	Cheek skin	2.6	2.7	2.1	2.6	1.6	2.3 ± 0.5	21.7
	Footpads	2.5	2.3	2.5	2.3	1.9	2.3 ± 0.4	17.4
SYN	Hard palate	1	1.5	2	1.4	1	1.4 ± 0.4	28.6
	Nasal planum	2	1	1.2	1.1	1	1.3 ± 0.4	31
	Cheek skin	1.1	1.3	1.1	1.6	1.7	1.4 ± 0.3	21
	Footpads	1	1	0	1	1	1 ± 0.3	30

Note: ATB = antibody; SD = standard deviation; CV = coefficient of variation.

Table 3. Individual and mean values (±SD) of immunoreactive MCs/cm of basal zone for each location with the antibodies used.

ATB	Locations	Animals 1	2	3	4	5	Mean ± SD
CK20	Hard palate	17	23	8	42	23	22.60 ± 12.46
	Nasal planum	31	17	13	46	22	25.80 ± 13.14
	Cheek skin	122	62	120	216	69	117.80 ± 61.56
	Footpads	21	17	7	10	16	14.20 ± 5.63
CK8 + 18	Hard palate	22	11	7	26	6	14.40 ± 9.07
	Nasal planum	19	10	13	39	9	18.00 ± 12.37
	Cheek skin	119	43	99	182	43	97.20 ± 58.19
	Footpads	16	9	3	5	9	8.40 ± 4.98
NSE	Hard palate	14	16	5	40	13	17.60 ± 13.20
	Nasal planum	20	14	10	47	16	21.40 ± 14.76
	Cheek skin	121	34	85	181	71	98.40 ± 55.70
	Footpads	21	21	0	12	20	14.80 ± 9.09
CGA	Hard palate	4	22	5	15	6	10.40 ± 7.83
	Nasal planum	18	17	3	38	8	16.80 ± 13.40
	Cheek skin	99	45	79	179	57	91.80 ± 52.96
	Footpads	14	19	2	12	18	13.00 ± 6.78
SYN	Hard palate	11	17	7	28	1	12.80 ± 10.30
	Nasal planum	18	4	5	28	3	11.60 ± 11.01
	Cheek skin	83	37	46	151	35	70.40 ± 49.04
	Footpads	3	10	0	1	3	3.40 ± 3.91

Note: ATB = antibody; SD = standard deviation.

immunoreaction was usually more intense for CK20 than for other antibodies tested. CV was ≤20% with the use of the anti-CK20 antibody, indicating that the results are more homogeneous than those obtained with any of the other antibodies tested. These findings are in agreement with the sensitivity and specificity for MCs' identification demonstrated for antibodies against CK20 in humans (Moll et al. 1995) and rodents (Moll et al. 1996).

The pattern of immunostaining in MCs was different for each antibody used. These differences of immunostaining are due to the intracellular location of the specific peptide against which each antibody is directed. Cytokeratins are integral constituents of the cytoskeleton in the mammalian MCs (Eispert et al. 2009; Halata et al. 2010). Immunoreactive products in the MCs labelled with anti-cytokeratin antibodies appear to be homogeneously distributed within the cytoplasm, matching with the typical arrangement of cytokeratins in the form of fine intermediate filaments distributed within the cell (Halata et al. 2010; Ramírez et al. 2015). In addition, being the structural cytoskeleton, CKs in the MCs seem to be participating in the cell plasticity necessary for mechanoreceptive function (Eispert et al. 2009). In the NSE-positive cells, the immunoreaction was dispersed throughout the cytoplasm in correspondence to the distribution of ribosomes and the rough endoplasmic reticulum observed in the MCs under electron microscopy (Gu et al. 1981; Vinores et al. 1984; Ramírez et al. 2015). It has been noted that the number of ribosomes per cell varies with the cell cycle (Darzynkiewicz et al. 1979); therefore NSE expression by MCs may be different depending on the cell status.

CGA is the main element of the soluble matrix protein in secretory granules of various neuroendocrine cells, including mammalian MCs, usually coexisting with other neuropeptides such as VIP, peptide histidine isoleucine, substance P, or CGRP in the same granule (Gauweiler et al. 1988; Hartschuh et al. 1989; Fantini & Johansson 1995). Using anti-CGA antibody, immunostaining in MCs of the dog was restricted to the cytoplasmic portion closest to the nerve ending where dense-core granules are concentrated (Ramírez et al. 2015). SYN, a specific component of the membrane of presynaptic vesicles in neurons and neuromuscular junctions (Ortonne et al. 1988), showed a cytoplasmic immunostaining pattern in the opposite cellular side in relation to CGA antibody, which is the part of the cytoplasm farthest from the nerve terminal. This particular distribution has been related with the structural distribution of small clear-content vesicles observed with an electron microscope (Ramírez et al. 2015). Secretory electron dense-core granules and small clear vesicles could be involved in two different secretory pathways of MCs in dogs, as appears to be in MCs of pig and neurons (Lundberg & Hokfelt 1983; García-Caballero et al. 1989). Two secretory pathways have been proposed in MCs: a Ca2+-dependent pathway that serves a mechanosensory function and neurotransmitter release, and a Ca2+-independent pathway that serves neuroendocrine functions and neuropeptide release (Boulais et al. 2009; Maksimovic et al. 2014). Numbers, sizes, and electron densities of the granules and clear vesicles can vary between MCs from the same or different locations in the same animal or between different individuals (Beiras et al. 1987; Ramírez et al. 2015). Although these antibodies can identify the MCs in the skin, it appears to be not completely adequate to perform extensive studies in cell density or distribution because MCs with scant amounts of granules or vesicles could be difficult to identify by immunohistochemistry. In fact, CGA and Syn showed the lower intensity of immunoreaction and lower amounts of MCs in serial sections.

In conclusion, the results of the present study highlight the need to validate human commercial antibodies available for identification of MCs for any particular immunohistochemical study of density or mapping of this cell population in veterinary species. Antibodies developed against CK20 appear to be the most appropriate to effectively recognize MCs in the dog skin.

Disclosure statement

No potential conflict of interest was reported by the authors.

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